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Revised Small Subunit rRNA Analysis Provides Further Evidence that Foraminifera Are Related to Cercozoa

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Abstract. There is accumulating evidence that the general shape of the ribosomal DNA-based phylogeny of Eukaryotes is strongly biased by the long-branch attraction phenomenon, leading to an artifactual basal clustering of groups that are probably highly derived. Among these groups, Foraminifera are of particular interest, because their deep phylogenetic position in ribosomal trees contrasts with their Cambrian appearance in the fossil record. A recent actin-based phylogeny of Eukaryotes has proposed that Foraminifera might be closely related to Cercozoa and, thus, branch among the so-called crown of Eukaryotes. Here, we reanalyze the small-subunit ribosomal RNA gene (SSU rDNA) phylogeny by removing all long-branching lineages that could artifactually attract foraminiferan sequences to the base of the tree. Our analyses reveal that Foraminifera branch together with the marine testate filosean *Gromia oviformis* as a sister group to Cercozoa, in agreement with actin phylogeny. Our study confirms the utility of SSU rDNA as a phylogenetic marker of megaevolutionary history, provided that the artifacts due to the heterogeneity of substitution rates in ribosomal genes are circumvented.

Key words: Foraminifera — Cercozoa — Eukaryotes — *Gromia oviformis* — Molecular phylogeny — Long-branch attraction — Small-subunit ribosomal DNA

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Introduction

Phylogenetic studies based on the small-subunit ribosomal RNA gene (SSU rDNA) have yielded a "classical" view of eukaryotic evolution, in which several assumed primitive, isolated lineages diverged very early, while other groups, supposedly more recent and apparently simultaneously diverging, cluster in the so-called eukaryotic crown (Sogin 1991; Knoll 1992). However, the primitive status of "early" Eukaryotes, as defined by SSU rDNA phylogenies, has been a subject of long debate (see, e.g., Sogin 1997). Protein-based phylogenies are now providing accumulating evidences that the distinction between "early" and "crown" Eukaryotes is no longer accurate, because many—if not all—"early" Eukaryotes are probably highly derived groups, which are artifactually attracted to the base of SSU rDNA trees by the long-branch attraction (LBA) phenomenon (Embley and Hirt 1998; Philippe and Adoutte 1998; Philippe et al. 2000). Most striking is the example of the Microsporidia, which have been clearly shown to be highly derived, parasitic members of the Fungi (Keeling and Doolittle 1996; Hirt et al. 1999; Keeling et al. 2000; see Van de Peer et al. [2000b] for a review).

Because protein data are still lacking for a large part of the known eukaryotic diversity, the phylogenetic position of many groups of protists remains

unclear. Among these groups, the Foraminifera are of particular interest, because their evolutionary history can be inferred from their exceptionally well preserved fossil record. Foraminifera are a large and morphologically diverse group of mainly marine, testate protists with typical filamentous, granuloreticulose pseudopodia (Lee et al. 2000). Molecular data from both large- and small-subunit rDNAs have suggested a very ancient origin for the group (Pawlowski et al. 1994, 1996, 1999). But as discussed before (Pawlowski et al. 1996; Sogin 1997), rRNA genes from the Foraminifera are highly divergent. Therefore, a very early branching of the group must be taken suspiciously.

A recent actin-based phylogeny of Eukaryotes showed a close relationship among the Foraminifera, *Cercomonas*, and *Chlorarachnion* (Keeling 2001), suggesting a possible inclusion of Foraminifera within Cercozoa. This group of "crown" Eukaryotes was recently described on the basis of molecular data (Bhattacharya et al. 1995). The Cercozoa consist of a diverse assemblage of organisms, such as *Cercomonas*-like amoeboflagellates, the euglyphid testate filose amoebae, the plasmodiophorid plant pathogens, and the chlorarachniophyte green amoebae (Cavalier-Smith 1998; see also Cavalier-Smith and Chao 1997; Atkins et al. 2000; Kühn et al. 2000; Bulman et al. 2001; Vickerman et al. 2002). Clear morphological evidence is yet lacking to support the Cercozoa, but a close relationship between *Cercomonas* and *Chlorarachnion* was also recovered on the basis of both alpha- and beta-tubulin phylogenies (Keeling et al. 1998, 1999).

A possibility to avoid LBA artifacts, and to find the true phylogenetic position of an assumed fast-evolving, derived group of Eukaryotes, is to suppress all other possibly fast-evolving taxa from the analyses and, as suggested by Swofford et al. (1996), to reconstruct a tree including only ingroup taxa. These two conditions are necessary in order to avoid the attraction of the tested group by both the sequences from the outgroup and any other fast-evolving taxa. If the group examined is really a primitive, independent lineage of Eukaryotes, then its position in such phylogenetic trees should be left unresolved, with no clear affinity for any group of crown Eukaryotes. But if it is actually a derived member of one of these crown clades, then one can hope that there is still enough phylogenetic signal in its SSU rDNA sequences so that its true phylogenetic position might be retrieved. This approach was used by Van de Peer et al. (2000b), which showed that when considering among-site rate variation, reanalysis of large-subunit rDNA sequences from "crown" Eukaryotes, including two sequences of Microsporidia, supports the placement of the latter within the Fungi. In this study, we use this approach in order to test if there is any support in SSU rDNA sequences for a cercozoan affiliation of Foraminifera.

Materials and Methods

The complete SSU rDNA sequences of 5 Foraminifera and 49 other Eukaryotes were manually aligned using the Genetic Data Environment software (Larsen et al. 1993), following the secondary structure models proposed by Neefs et al. (1993) and Wuyts et al. (2000). Sequences were chosen so that representatives of all well-defined domains of the "crown" were included in the data set, using the studies by Van de Peer and De Wachter (1997) and Van de Peer et al. (2000a) as references. Eukaryotic lineages that were excluded from our data set comprise all groups (Diplomonadida, Parabasalia, Microsporidia, Euglenozoa, Heterolobosea, Radiolaria, Mycetozoa, Entamoebidae, and Pelobiontida) that are thought to have undergone a process of accelerated rate of evolution and, thus, may act as long branches in phylogenetic analyses (see, e.g., Stiller and Hall 1999; Morin 2000; Philippe and Germot 2000; Bolivar et al. 2001; López-Garcia et al. 2002). Relative rate tests performed with RRTree (Robinson-Rechavi and Huchon 2000) confirmed at both 5 and 1% levels that the representatives of these groups display significantly higher rates of substitution compared to crown species. The names, phylogenetic position, and GenBank accession numbers of the sequences used in this study are indicated in Table 1.

Evolutionary trees were inferred using the neighbor-joining (NJ) method (Saitou and Nei 1987), the maximum-parsimony (MP) method, and the maximum-likelihood (ML) method (Felsenstein 1981). The reliability of internal branches was assessed using the bootstrap method (Felsenstein 1985), with 1000 replicates for NJ analyses, 500 replicates for MP analyses, and 100 replicates for ML analyses. The Phylo_Win program (Galtier et al. 1996) was employed for distance computations and NJ building and bootstrapping, using the HKY85 model of substitution (Hasegawa et al. 1985). MP and ML analyses were performed with PAUP* (Swofford 1998). The most parsimonious trees for each MP bootstrap replicate were determined using a heuristic search procedure with 10 random-addition-sequence replicates and tree bisection–reconnection branch-swapping. The transversions cost was set to twice the transitions cost. For ML analyses, the TrN model of substitution was used (Tamura and Nei 1993), taking into account a proportion of invariable sites, and a gamma-shaped distribution of rates of substitution among sites, with eight categories. All necessary parameters were estimated from the data set using Modeltest (Posada and Crandall 1998). Starting trees of ML searches were obtained via NJ and swapped with the tree bisection–reconnection algorithm.

Results and Discussion

All methods of tree reconstruction yielded the same result: when no other sequences with particularly high rates of substitutions are included in the analyses, the Foraminifera branch together with the marine testate filosean *Gromia oviformis* as a sister group to the Cercozoa (Fig. 1). Bootstrap support values for the clade consisting of Foraminifera + *Gromia* + Cercozoa are good: 88, 94, and 88%, for ML, NJ, and MP analyses, respectively. As the ML tree in Fig. 1 contains only ingroup sequences, it is presented in an unrooted format, with a basal trichotomy separating

Table 1. List of the taxa used in our analyses

Taxonomic position	Species name	GenBank accession no.
Amoebozoa	*Acanthamoeba castellanii*	M13435
	Echinamoeba exundans	AF293895
	Hartmannella vermiformis	M95168
	Leptomyxa reticulata	AF293898
Opisthokonta	*Spizellomyces acuminatus*	M59759
	Schizosaccharomyces pombe	X58056
	Amanita muscaria	AF026631
	Nucleaaria simplex	AF349566
	Diaphanoeca grandis	L10824
	Leucosolenia sp.	AF100945
	Trichoplax adhaerens	L10828
	Mytilus edulis	L33448
Cryptophyta	*Goniomonas truncata*	U03072
	Guillardia theta	X57162
Glaucophyta	*Cyanophora paradoxa*	X68483
Rhodophyta	*Flintiella sanguinaria*	AF168621
	Compsopogon coeruleus	AF087128
	Glaucosphaera vacuolata	AB045583
Viridiplantae	*Dunaliella salina*	M84320
	Mesostigma viride	AJ250108
	Huperzia phlegmaria	X81964
	Helianthus annuus	AF107577
Alveolata	*Blepharisma americanum*	M97909
	Oxytricha nova	X03948
	Colpoda inflata	M97908
	Perkinsus marinus	AF126013
	Crypthecodinium cohnii	M64245
	Prorocentrum concavum	Y16237
Stramenopiles	*Cafeteria roenbergensis*	L27633
	Thraustochytrium multirudimentale	AB022111
	Blastocystis hominis	U51151
	Apodachlya brachynema	AJ238663
	Chrysolepidomonas dendrolepidota	AF123297
	Ectocarpus siliculosus	L43062
Haptophyta	*Coccolithus pelagicum*	AJ246261
	Phaeocystis cordata	AF163147
	Pavlova salina	L34669
Cercozoa	*Gromia oviformis* 1	AJ457811
	Gromia oviformis 2	AJ457812
	Cercomonas longicauda	AF101052
	Euglypha rotunda	X77692
	Pseudodifflugia cf. *gracilis*	AJ418794
	Nucleaaria-like filose amoeba NPor	AF174374
	Massisteria marina	AF174374
	Chlorarachnion reptans	U03477
	Lotharella vacuolata	AF054890
	Plasmodiophora brassicae	U18981
	Spongospora subterranea	AF310899
	Phagomyxa odontellae	AF310904
Foraminifera	*Allogromia* sp.	X86093
	Astrammina rara	AJ318223
	Reticulomyxa filosa	AJ132367
	Trochammina sp.	X86095
	Bolivina spathulata	AJ318227

Opisthokonta, Amoebozoa, and Bikonta, following a recent suggestion of Stechmann and Cavalier-Smith (2002). The stem branch leading to the Foraminifera was reduced to half of its actual size to make the rest of the tree clearer, which emphasizes the high divergence of foraminiferan SSU rDNA sequences. However, their inclusion in the analyses does not seem to disrupt the global phylogenetic informativeness of the molecule, because all groups of "crown" Eukaryotes that are now firmly established on the basis of molecular data (see, e.g., Van de Peer et al. 2000a) are recovered with confident bootstrap support.

Given the fact that highly diverging outgroup sequences generally join the longest branch in the ingroup (Wheeler 1990), the position of the Foraminifera in the tree shown in Fig. 1 might be regarded as the result of the artificial clustering of the foraminiferan sequences to one of the most rapidly evolving group of "crown" Eukaryotes, i.e., the Cercozoa. In order to test this hypothesis, we performed additional analyses on two enhanced data sets, incorporating sequences that were primarily rejected by relative rate tests. The first data set included some more diverging members of the Amoebozoa, like Mastigamoebidae, and the second one included some rapidly evolving members of the polycystine Radiolaria. In both cases, the phylogenetic position of the Foraminifera remained unchanged in ML analyses (data not shown). These results show that Foraminifera branch with Cercozoa even in the presence of some faster-evolving lineages, excluding the hypothesis that their grouping together in Fig. 1 was an artifact. Besides, analyses of the first data set showed that the ML topology is even recovered with NJ analyses, provided that enough slowly evolving members of the Amoebozoa are included (data not shown). Furthermore, ML analyses of the second data set interestingly revealed that the Radiolaria might represent a sister group to the clade comprising Foraminifera, *Gromia*, and Cercozoa, as already hypothesized by Burki et al. (2002). In this case, however, NJ analyses failed to recover the sister-group relationship between *Gromia* and the Foraminifera, and the latter were attracted to the fast-evolving polycystine Radiolaria in the NJ tree (data not shown).

The fact that Foraminifera and Cercozoa branch together even in the presence of some faster-evolving lineages suggests that a true phylogenetic signal for this relationship exists in SSU rDNA sequences. Indeed, a screening of the primary structure of the sequences used in our analyses revealed that seven of the eight nucleotide states that specifically define the Cercozoa (i.e., that are absent in all other crown Eukaryotes) are also present in the foraminiferan sequences. Besides, Foraminifera and Cercozoa share the same character states in most other informative but potentially homoplasic positions, i.e., where other groups or subgroups of crown Eukaryotes also display the same nucleotide. Interestingly, these

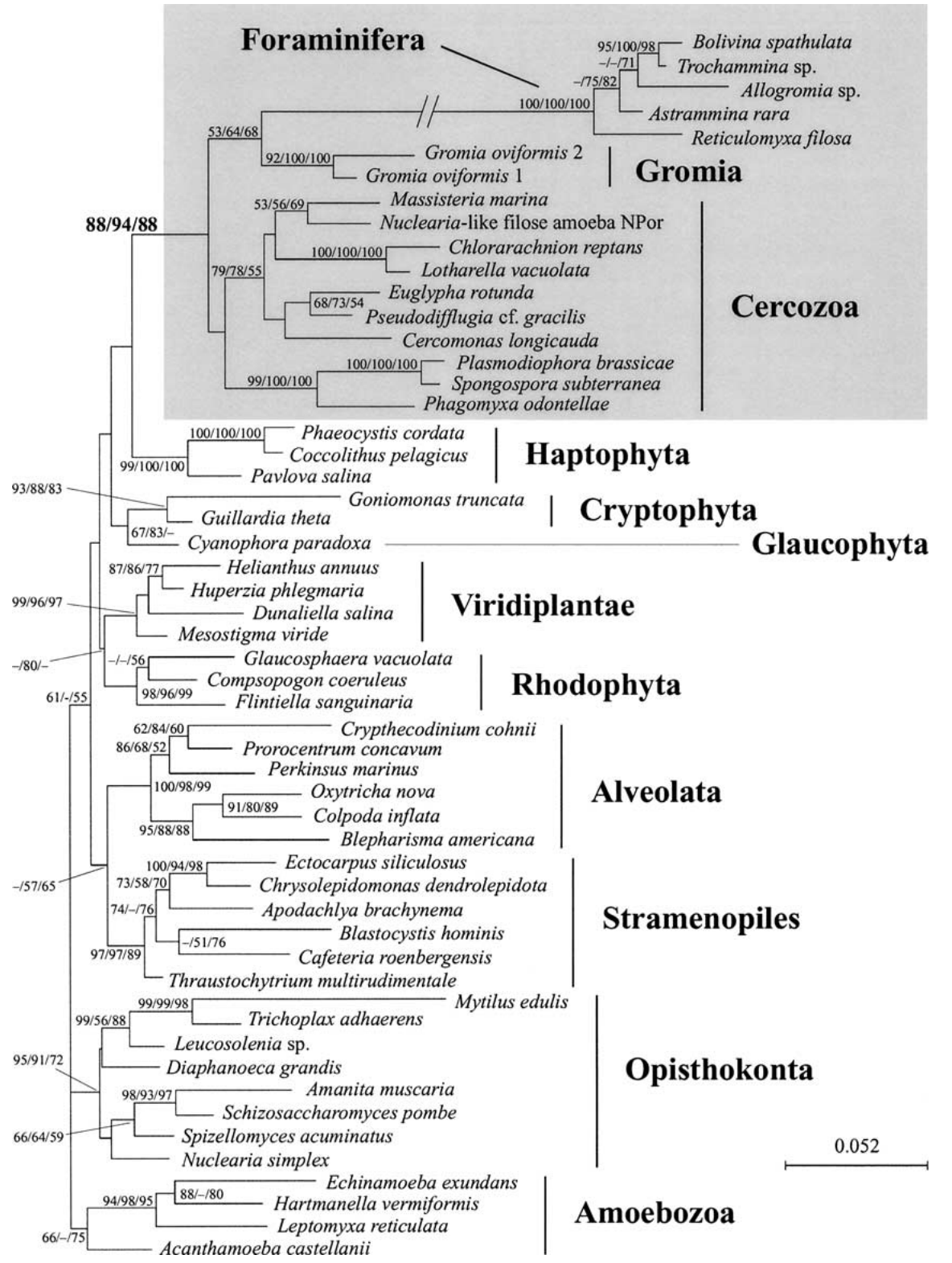

Fig. 1. Phylogenetic position of the Foraminifera based on the analysis of 54 SSU rDNA sequences from diverse Eukaryotes, using 1117 unambiguously aligned positions. The tree is the ML tree, which is presented in an unrooted format (see text); its log likelihood value is −14,413.669. When no other sequences with high rates of substitution are included in the analyses, the Foraminifera cluster with the Cercozoa, as a sister group to the marine testate filosean *Gromia oviformis* (*shaded area*). Numbers at internal nodes are the bootstrap support values for ML, NJ, and MP analyses after 100, 1000, and 500 replicates, respectively; *dashes* indicate values under 50%. When all three bootstrap support values were inferior to 50%, they are not indicated. All branches are drawn to scale, except the stem-branch leading to the Foraminifera, which was reduced to half its actual size.

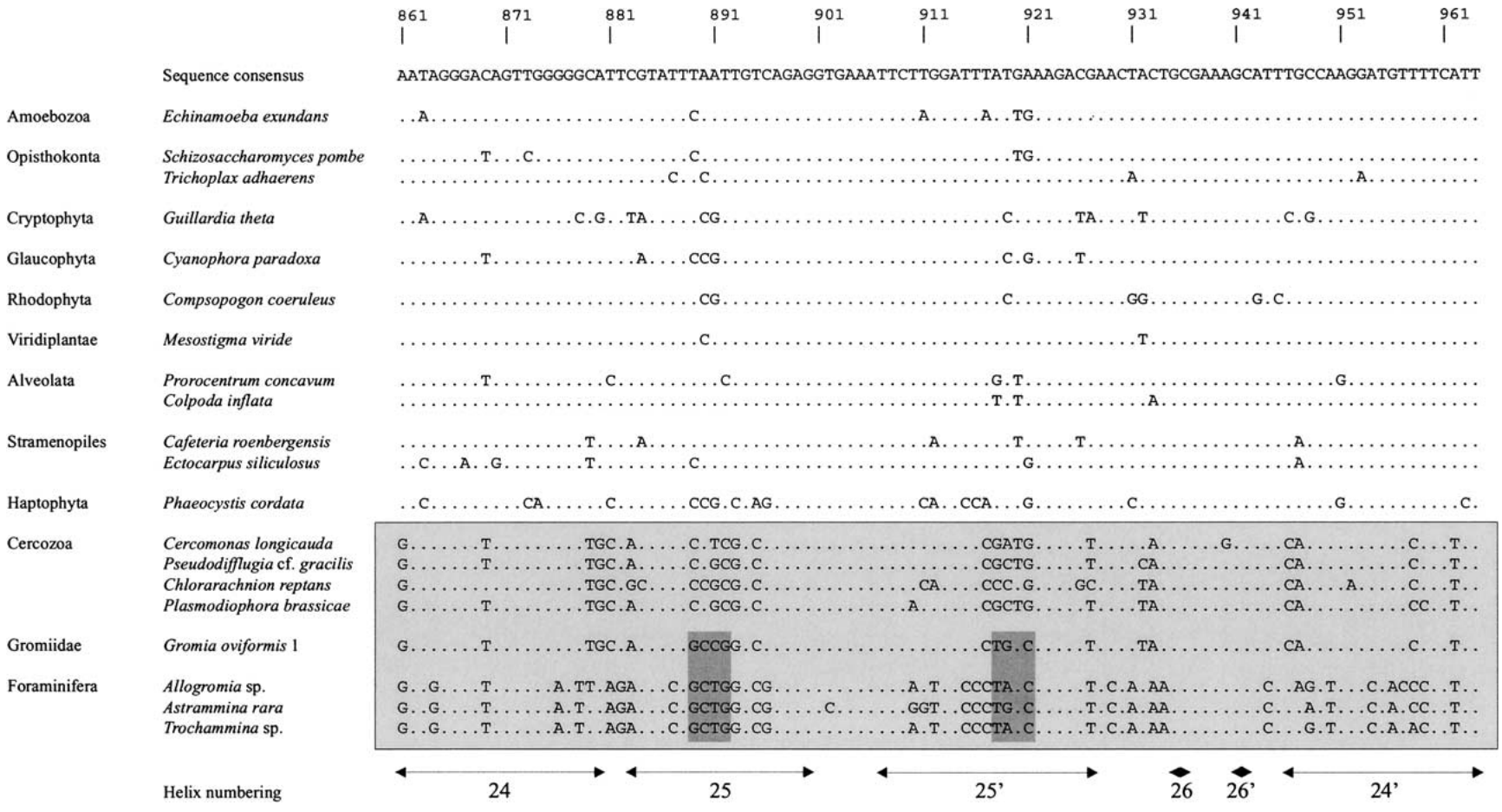

Fig. 2. Alignment of helices 24 to 26 of the SSU rDNA sequences of 4 Foraminifera and 16 "crown" Eukaryotes, highlighting some of the specific positions that indicate a close relationship among Cercozoa, Foraminifera, and the testate marine filosean *Gromia oviformis* (*light gray box*). The *dark gray boxes* show two complementary sequences signatures (GCYG and TRGC) uniting Foraminifera and *G. oviformis*. *Dots* indicate identity with the consensus sequence, which was defined as the most frequent nucleotide in each position. The limits of each helix, as defined by Wuyts et al. (2000), are indicated below the alignment. Numbers at the top refer to the sequence of *Cercomonas longicauda* (GenBank accession number AF101052).

cercozoan-specific signatures are not completely randomly scattered through the SSU rDNA, but are concentrated mainly in the region comprising helices 24 to 26 (Fig. 2), suggesting local rearrangements of the secondary structure of the molecule. In our opinion, this feature is not likely to be due to random convergence, because in that case one might expect the foraminiferan sequences to show similar convergences toward the specific signatures of any other group of crown Eukaryotes, which is not the case.

The preservation of cercozoan-specific nucleotides in Foraminifera is particularly significant, given the high divergence of the foraminiferan stem lineage, as illustrated by about 130 specific nucleotide sites which define this group in the conserved regions of our alignment. Analyses of partial SSU rDNA sequences from a large sampling of extant foraminiferan lineages, using calibration points obtained from the rich fossil record of the group, revealed that an episodic change of substitution rates occurred in the stem lineage leading to the Foraminifera. According to our calibration, the rate of substitution in the most conserved regions of the SSU rDNA averaged at least 1.0 to 1.65 substitutions/1000 sites/10^6 years during the stem lineage evolution of Foraminifera. This rapid burst of the evolutionary rate was followed by a return to a "normal" value of about 0.03 substitutions/1000 sites/10^6 years during the subsequent radiation of the different foraminiferan lineages (Pawlowski and Berney, unpublished), comparable to evolutionary rates calculated in other groups of Eukaryotes (see, e.g., Sorhannus 1996).

Our data confirm a recent analysis of actin sequences (Keeling 2001) and are also congruent with unpublished analyses of RNA polymerase II coding sequences (Longet, personal communication). All these studies clearly show that Foraminifera are related to Cercozoa, a relationship that is also strongly supported by polyubiquitin structure (Archibald, personal communication). However, whether Foraminifera are a derived lineage of Cercozoa or their sister group remains disputable. Although the Foraminifera and *Gromia oviformis* appear as sister groups to Cercozoa in the ML tree (Fig. 1), this is not the case in other analyses. In both the NJ tree and one of the two most parsimonious MP trees, the clade comprising Foraminifera and *G. oviformis* formed a sister group to the plasmodiophorid plant pathogens (data not shown), proning the inclusion of both *Gromia* and the Foraminifera within the Cercozoa.

The close relationship between Foraminifera and *G. oviformis* suggested by our analyses (Fig. 1) could argue in favor of a cercozoan origin of Foraminifera. In fact, for a long time *G. oviformis* was considered a member of the Testaceafilosia (class Filosea), whose representatives (e.g., *Euglypha*, *Paulinella*) branch within Cercozoa in molecular analyses (see, e.g., Cavalier-Smith and Chao 1997; Cavalier-Smith 1998). However, recent analyses of SSU rDNA sequences of *Gromia* (Burki et al. 2002) showed that this genus is not closely related to the Euglyphida (*Euglypha*, *Paulinella*) but, rather, branches near the base of the cercozoan clade. This may suggest that the common ancestor of Foraminifera and *Gromia* evolved from the cercozoan stem lineage before the radiation of the living representatives of the Cercozoa. Further studies of protein-coding genes should allow resolution of this problem, as well as attainment of a clearer idea of the true evolutionary relationships among the different cercozoan lineages.

Our findings bring further support to the idea that the so-called eukaryotic crown is actually an artifactual clustering of slowly evolving sequences, to the exclusion of a few rapidly evolving lineages (Stiller and Hall 1999; Philippe et al. 2000). The relationship between Foraminifera and Cercozoa also suggests that although diversity within the protists remains largely underestimated (see, e.g., Moreira and López-Garcia 2002), most eukaryotic lineages might have evolved from a reduced number of large "supergroups." Finally, our results show that even if recent protein-based analyses have cast strong doubts on the phylogenetic potential of SSU rDNA sequences to recover deep evolutionary relationships (see, e.g., Baldauf et al. 2000; Moreira et al. 2000), SSU rDNA data might still remain a source of valuable phylogenetic information. Although protein data are rapidly accumulating, they are available for a very limited taxonomic sampling of unicellular Eukaryotes. Because of technical constraints, the evolutionary studies of many noncultivable protozoans, such as Foraminifera and Radiolaria, remain largely dependent on ribosomal genes phylogenies, hence their crucial importance in megaevolutionary studies.

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